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**The Kaposi's sarcoma-associated herpesvirus ORF57 protein: a pleurotropic regulator of gene expression**

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Running Head: **Viral mRNA export by KSHV ORF57**

**Abstract**

*Herpesviridae* comprises over 120 viruses infecting a wide range of vertebrates including humans and livestock. Herpesvirus infections typically produce dermal lesions or immune cell destruction, but can also lead to oncogenesis, especially with Kaposi's sarcoma-associated Herpesvirus (KSHV). All herpesviruses are nuclear replicating viruses that subvert cellular processes such as nucleo-cytoplasmic transport for their advantage. For virus replication to take over the cell and produce lytic infection requires that virus gene expression outpace that of the host cell. KSHV ORF57 appears to play a major role in this by 1) serving as a nuclear export receptor to carry intronless viral mRNAs out of the nucleus and 2) inhibiting expression of intron-containing host mRNAs. As the majority of virally encoded mRNAs are intronless compared to host cell mRNAs, these two mechanisms are critical to overcoming host gene expression.

**Key Words:** human Herpesvirus, immediate early protein, nuclear transport, viral mRNA export, Kaposi's sarcoma-associated Herpesvirus ORF57, Herpes simplex virus ICP27

**Abbreviations used:** IE, immediate early; KSHV, Kaposi's sarcoma-associated herpesvirus; HSV-1, Herpes simplex virus type 1; NES, nuclear export signal; NLS, nuclear localization signal; LMB, leptomycin B

## Introduction

The *Herpesviridae* comprises over 120 viruses infecting invertebrate and vertebrate organisms including oyster, fish, amphibians, reptiles, birds, and mammals including humans and livestock (1). Their involvement in a range of prominent medical and veterinary diseases makes them one of the most important virus families. Herpesviruses have large enveloped virions containing double-stranded linear DNA genomes. They vary greatly in their pathology and biology but all, following primary infection, establish lifelong latent infections, which can recrudesce to cause recurrent disease. Individual Herpesviruses are well adapted to their specific hosts, and primary or recurring infections frequently are inapparent. Under certain circumstances, particularly in immuno-suppressed individuals, Herpesviruses can be life-threatening. Herpesviruses have also been implicated in various types of cancer (2).

Eight human Herpesviruses (HHVs 1-8) have been identified so far, which are categorized into three subfamilies (alpha-, beta- and gamma- *herpesvirinae*). The eight HHVs are: herpes simplex virus (HSV) types 1 and 2, varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), HHV-6, HHV-7 and Kaposi's sarcoma-associated Herpesvirus (KSHV/HHV-8) (reviewed in 3). KSHV is the most recently identified herpesvirus infecting humans and is associated with multicentric Castleman's disease, a rare type of B-cell lymphoma called primary effusion lymphoma, and a major neoplasm of Acquired Immune Deficiency Syndrome (AIDS) patients called Kaposi's sarcoma (4).

Like other Herpesviruses, KSHV has two distinct states: it can remain in a low activity persistent state generally referred to as latency or engage in a highly active destructive lytic replication cycle. Herpesvirus gene expression and viral replication is a complex, tightly regulated process involving four distinct stages of viral gene transcription: latent, immediate early (IE), early, and late. Expression of certain proteins of the IE class acts as the switch for transition from the latent state to the lytic replication cycle. These IE proteins do not require cellular protein synthesis for their transcription, but in order to achieve a high level of replication during lytic infection the virus requires use of many aspects of the host cell gene expression machinery. One way the virus can compete with the host cell substrates for this machinery is taking advantage of the fact that few herpesvirus genes compared to host cell genes contain introns. The expression of these intronless viral genes is preferentially facilitated by a KSHV protein called ORF57 (also known as the KS-SM, or Mta protein). ORF57 is a *trans*-acting multifunctional regulatory protein that enhances expression of intron-lacking viral genes and is involved in the export of viral mRNAs. It is highly conserved within the *herpesviridae*, having homologues in all three subfamilies (alpha, beta and gamma) from a variety of host species. All these homologue proteins affect viral and

cellular gene expression, but variations have been observed among different Herpesviruses for the precise mechanisms. This review will discuss the mechanisms utilised in infection by KSHV ORF57 and its homologues from other herpesviruses.

### **General features of ORF57**

The presence of an ORF57 homologue in every sequenced herpesvirus of mammals and birds suggests that its regulatory role is maintained and essential: in fact deletion of ORF57 completely blocks production of progeny virions in KSHV (5). Characterized ORF57 homologues in humans include HSV-1 ICP27 (6, 7), EBV Mta (also known as BMLF1, M, SM, or EB2) (8), HCMV UL69 (9), VZV ORF4 (10), and in other primates herpesvirus saimiri (HVS) ORF57/IE52 (11). Overall, the identity at the amino acid level is approximately 30% among the gammaherpesvirus ORF57 homologues from KSHV, EBV and HVS. Latent state of infection of KSHV in BCBL-1 cells can be activated to a lytic cycle by treatment with phorbol esters. KSHV ORF57 is expressed between 2h- 4h after treatment, immediately following the appearance of transactivator ORF50 but prior to other early gene products (12, 13). The ORF57-null KSHV recombinant was unable to produce virion progeny or fully express several other lytic KSHV genes, except when ORF57 was provided in *trans* (5). The 455 amino acid long ORF57 protein is expressed very early in infection from a 1.7-kb spliced RNA bearing several in-frame ATG codons (14-16).

### **Effect of ORF57 on viral and cellular gene expression**

The homologous HSV-1 ICP27, EBV Mta, and HVS ORF57 genes all localize to the nucleus at steady state and activate expression of other genes via post-transcriptional mechanisms (6, 17-19). Some homologues have been shown to act post-transcriptionally affecting RNA splicing and transport. Other functions include downregulation of intron-containing transcripts and upregulation of certain late messages.

The KSHV ORF57 protein is a transactivator that increases expression of heterologous genes both transcriptionally and post-transcriptionally using various reporter assays (15, 16). The increase in activity was irrespective of whether the reporter was upstream to promoters from different viruses (15); however activation did not occur for all promoters tested (16). Thus ORF57 is not a broad-spectrum activator such as HSV ICP0. Though ORF57 homologues are known to enhance cytoplasmic transport of mRNA this increase in reporter activity may also be transcriptional because nuclear levels of reporter mRNA also increased.

KSHV ORF57 is specific in the substrates for which it regulates expression. The accumulation of some viral RNAs under control of the HCMV IE promoter were strongly augmented while others were unaffected, indicating that post-transcriptional upregulation by ORF57 is transcript specific (16). The activating effect of HSV-1 ICP27 and EBV Mta is also transcript specific, not activating all viral genes equally (20, 21).

ORF57 synergistically augments the effect of the KSHV major transactivator, ORF50 on several KSHV promoters (16). This effect could reflect action at multiple levels including post-translational enhancement of ORF50 as ORF57 has been shown to directly interact with ORF50, to stimulate its transcriptional activity, and synergy is promoter-specific (13, 16).

The cumulative effect of ORF57 and its homologues on expression of a specific gene likely depends on multiple factors, including the sequence of the gene itself, the presence or absence of introns as well as the sequence of the 3' untranslated region and the coding region. ORF57 may play an important role in the activation of other viral lytic genes, particularly those that are expressed as unspliced mRNAs, to enhance the lytic cascade.

### **ORF57 and splicing factors, effects on host cell splicing**

KSHV ORF57 protein fused to GFP exhibited a punctate nuclear distribution that co-localised with the cellular splicing factor SC-35 (14), consistent with similar observations for HSV-1 ICP27 (22). When the activity from intron-containing and intronless reporter constructs was compared in the presence of KSHV ORF57, a slight decrease in reporter activity with intron-containing reporter constructs was observed (15). Thus introns in the target gene appear to interfere with ORF57-mediated activation, an effect that may be important in selectively enhancing expression of certain KSHV genes, which are intronless. However the inhibition was not marked as seen with EBV Mta (23). HSV-1 ICP27, HVS ORF57, and EBV Mta inhibit the expression of genes containing introns, perhaps due to interference with the normal processing of intron-containing pre-mRNAs (reviewed in 24, 25, 26).

The role of ORF57 in cellular gene regulation is further supported by evidence for a direct physical interaction between this protein and mRNA processing factors. Several regions of KSHV ORF57 protein involved in interactions with cellular RNA processing and translation factors have also been roughly mapped. Residues at the N- and C-terminus bind hnRNP K and at the C-terminus bind protein kinase CK2  $\alpha'$  (27). Additionally, the middle region of ORF57 interacted with PCBP1, a cellular RNA binding protein involved in IRES-mediated gene expression (28).

In keeping with these experimental observations there are indications that ORF57 might also enhance expression of specific cellular genes (15). The data available to date argues against an ORF57-encoded activity that globally impairs splicing or actively represses

expression from intron-containing genes. However it does not exclude the possibility that some viral genes might display intron-dependent responses to ORF57 (16).

### **KSHV ORF57 nucleocytoplasmic shuttling and viral mRNA export**

Whereas many RNA viruses replicate entirely in the cytoplasm where they first enter the cell, herpesviruses must move DNA, RNA and proteins between the cytoplasm and nucleus for infection. This requires utilizing the host nuclear pore complexes, large megadalton multi-protein complexes that regulate transport of macromolecules in and out of the nucleus (29).

Expression of KSHV ORF57 led to an increase in cytoplasmic CAT poly (A)+ mRNA versus an increase in nuclear CAT poly (A)+ mRNA, suggesting that ORF57 may facilitate nucleocytoplasmic transport of target mRNAs (15). Unlike with EBV Mta (30), no cytoplasmic translocation of ORF57 was observed upon overexpression of the cellular export receptor protein CRM1 with ORF57 (15). Full-length ORF57 exhibited a punctate nuclear distribution but an N-terminal deletion exhibited a predominantly cytoplasmic distribution, indicating that a nuclear localisation signal (NLS) resides between amino acids 1-180 (14). Very recently, three separate NLSs were confirmed in this region and disruption of any of the three prevented localisation of ORF57 in the nucleus (31). Wild-type ORF57 and its homologues HSV-1 ICP27 and EBV Mta exhibit a nuclear speckled pattern consistent with its general accumulation and steady-state localization in the nucleus (15, 16, 32). The exact mechanism of ORF57 import into the nucleus is not defined. It is not known whether it follows the classical importin- $\alpha$ ,  $\beta$  import pathway by binding initially to importin- $\alpha$ , which in turn binds to importin- $\beta$ , or if it binds directly to importin- $\beta$ , or if it employs an as yet unidentified receptor for viral protein import. ICP27 import was blocked by addition of the importin- $\beta$  binding domain peptide of importin- $\alpha$  in *Xenopus* oocytes (33).

ORF57 can shuttle between the nucleus and cytoplasm in a heterkaryon assay (14), but unlike HSV-1 ICP27 (32, 34) does not shuttle in the presence of actinomycin D (35). This indicates a further variation in specific mechanism and function between these two homologues. Moreover, it suggests that in the case of ORF57 an mRNA substrate is necessary to trigger an additional shuttling role in mRNA export. In this regard ORF57 probably acts similarly to host cell nuclear transport receptors. In support of this role, HVS ORF57 has been shown to bind the host nuclear transport receptors importin- $\alpha$  1 and 5 (36) that can associate with both import and export factors. No classical nuclear export signal (NES) has been identified for KSHV ORF57 and its export is not dependent on the principle cellular *protein* export receptor CRM 1 (37) and is insensitive to Leptomycin B (LMB), a CRM1 inhibitor (38). Instead the ORF57 N-terminus bound directly to the host export factor REF/Aly and export receptor TAP (the principle cellular *mRNA* export receptor) in the virus-infected cells

(38). This same region could also bind to protein kinase CK2  $\beta$  (27), providing a possible mechanism for regulating binding to the export machinery as phosphorylation has been shown for other proteins to regulate their binding to transport receptors.

KSHV ORF57 mediated mRNA export appears to be quite complex and many aspects are as yet not understood. Its role as an export factor favors export of unspliced transcripts (38). This may be due to specificity in binding substrates, which could increase the likelihood of intronless viral transcripts binding to cellular transport receptors that normally recognize substrates having splicing/ exon junction complex factors bound to them. However, the export route of many intron-containing KSHV RNA transcripts is not clear. In HSV-1 ICP27 an RNA-binding RGG box has been defined. Sequence homology shows an R and G rich region also in KSHV ORF57, though its function has not yet been tested in KSHV and whether it preferentially binds intronless viral mRNAs or both intronless and intron-containing equally. Details of many aspects of export by ORF57 homologues nonetheless vary considerably between different viruses. Unlike KSHV ORF57, HSV-1 ICP27 has a classic leucine-rich NES, but it also utilizes the REF/ TAP-mediated export pathway employed by most cellular mRNAs (33, 39). There are conflicting reports about whether export is CRM-dependent or -independent for the EBV SM protein, although it has been shown to bind REF and TAP (30, 40). An NES has been identified for HCMV UL69, the disruption of which blocks export; however, it is unusually long and the receptor has yet to be identified. UL69 binds UAP65 and URH49, cellular DEXD/H-box RNA helicases involved in export, but unlike ORF57 no direct interaction occurred between UL69 and REF in UAP56- or URH49-containing complexes (41).

### **Putative functional domains of ORF57 protein**

The domain organization of ORF57 has only partly been directly mapped. Some of its organization is inferred from what has been mapped for its homologues. On the basis of amino acid homology with its herpesvirus counterparts several putative domains are present (Fig. 1). The equivalent of the mRNA-binding RGG box mapped for HSV-1 ICP27 (42) is thought to be located at a series of Arg-Gly dipeptides in ORF57 (43); however, in the same general region of the protein there are two Arg-X-Pro tripeptide type domains and an Arg-Pro rich region that could also provide this function (Fig. 1). The export factor REF bound strongly to the N-terminus of ORF57 that includes these arginine-rich regions, but the minimum region required was at the end of this segment (aa 181-215) so that mRNA substrates of ORF57 could be bound coincidentally with the export receptor to facilitate their export from the nucleus (38).



ORF57 does not seem to contain a classical leucine-rich NES potentially capable of binding CRM1 as found in EBV Mta, HVS ORF57 and HSV ICP27 (11, 30, 44). The ICP27 NES occurs at the N-terminus, but the only moderate homology to a classical-type NES in ORF57 occurs at the C-terminus. Regions involved in import were recently mapped also to the ORF57 N-terminal region where three NLSs have been confirmed (31) (Fig. 1).

The C-terminal and middle region contains a greater degree of similarity amongst the ORF57 homologues (45). A zinc finger motif in the C-terminal region of HVS ORF57 and HSV-1 ICP27 is required both for transactivation and repression (46, 47). Mutation analysis demonstrated that the C-terminus of ICP27 encodes a zinc finger motif that resembles protein:protein interaction domains (48). Self-interaction of ICP27 requires the residues that make up the zinc finger (49). The KSHV ORF57 C-terminal region is also involved in self-interaction (27). Although they have not been functionally characterized, two putative zinc finger-like motifs occur in the C-terminal domain of ORF57 based on amino acid similarity (48). Moreover a hydrophobic GLFF domain, highly conserved in gamma-herpesviruses also occurs in the C-terminal region. Unlike its homologues in other herpesviruses, ORF57 contains a leucine zipper motif (generally found in DNA binding proteins) in the C-terminal region with a possible role in self-interaction or DNA binding (50) (Fig. 1).

Its ability to redistribute snRNPs was the first indication that ORF57 functions in inhibiting splicing. The HSV-1 ICP27 C-terminal region is required for redistribution of snRNPs and in anti-Sm (an SR-splicing factor protein family) sera co-immunoprecipitations the region required for SR-proteins co-immunoprecipitation with ICP27 was aa 450-504 (51). Similarly in HVS ORF57 the zinc finger-like domain is required for the intense SC-35 nuclear staining (46).

### **Alternative export pathways employed by Herpesviruses**

The variability in mapping of NES/ NLS sequences and transport factor binding suggests that multiple pathways may be employed by herpesviruses for export of viral mRNAs and proteins. There are also several experimental arguments supporting this idea: (i) unlike in yeast, REF is dispensable in metazoan cells (52, 53). (ii) An ICP27 HSV-1 deletion mutant (d3-4) incapable of binding REF is fully replication competent (54), although the mutant protein is defective in its export ability (33). As export of viral messages is essential for replication, this indicates use of an alternative pathway for mRNA export by the mutant virus. Moreover it has been shown recently that wild-type HSV-1 and ICP27 mutant d3-4 virus are both sensitive to LMB for growth (55). Because LMB has no known targets other than CRM1, it was presumed that the LMB sensitivity of HSV-1 reflects a need by the virus to utilize CRM1 during its lytic replication. As mutations in ICP27 confer LMB resistance, CRM1 and ICP27 clearly must have an important relationship and may participate in a

common biological process (55). (iii) KSHV ORF57-mediated mRNA export, although CRM1-independent, reduced export mediated by the human immunodeficiency virus type 1 export factor Rev which is entirely CRM1 dependent. Thus the export routes employed by both export factors may converge at some common point, suggesting a requirement for common transport apparatus components such as nucleoporins (38).

The means by which herpesvirus gene expression can effectively outcompete host cell gene expression may thus be due to several factors. Multiple export pathways subverted by viral proteins can elevate the number of viral mRNAs that are exported. Additionally ORF57 increases viral mRNA export presumably by increasing access of intronless mRNAs (mostly viral) for the cellular transport machinery that preferentially recognizes mRNAs bound by exon junction complex proteins. ORF57 and its homologues disruption of host cell splicing results in the degradation or inhibition of host cell mRNAs early on in the infection process (56). Finally, the U<sub>L</sub>41 gene product, virion host shut off (vhs, 57, 58) leads to the endonucleolytic cleavage of host mRNAs while at the same time temporarily evading the apoptotic cellular response to infection. Together these virus activities converge to give the virus a kinetic edge over the host cell in gene expression.

Interestingly although ORF57 homologues are conserved in sequence and domain structure and behave similarly in assays for reporter gene activation, their level of functional conservation does not enable them to cross-complement one another (5, 59, 60). Thus the different viruses may use distinct nuclear export receptors unique to their particular host cells. This may contribute to the different host cell tropism of these viruses if a specific combination allows them to kinetically outcompete host cell transcription/ translation.

### **Conclusions and Future prospects**

We postulate that initially in Herpesvirus infection, when the immediate early virus genes are beginning to express, the virus utilizes cellular machinery for viral mRNA export with ORF57 acting as an export factor to recruit REF and TAP proteins. Additionally, ORF57 and its homologues suppress cellular gene expression by disrupting splicing and the vhs equivalent function degrades host cell mRNAs. At intermediate times in infection viral mRNAs are expressed in abundance and compete with cellular mRNAs for export. By the later stages of infection, ORF57 protein family members may entirely take over the role of host transport receptors for transporting viral mRNAs out of the nucleus. Expression of certain late genes is heavily dependent upon the presence of ORF57 or its homologues (Fig. 2). Thus far the only measure of specificity for mRNA substrates of ORF57 has been the presence or absence of introns. Future work may identify specific structural elements or cis-regulatory sequences that give further specificity to the RNA targets of ORF57 or differences in transport receptors and other host partners. These types of viral-host interactions may

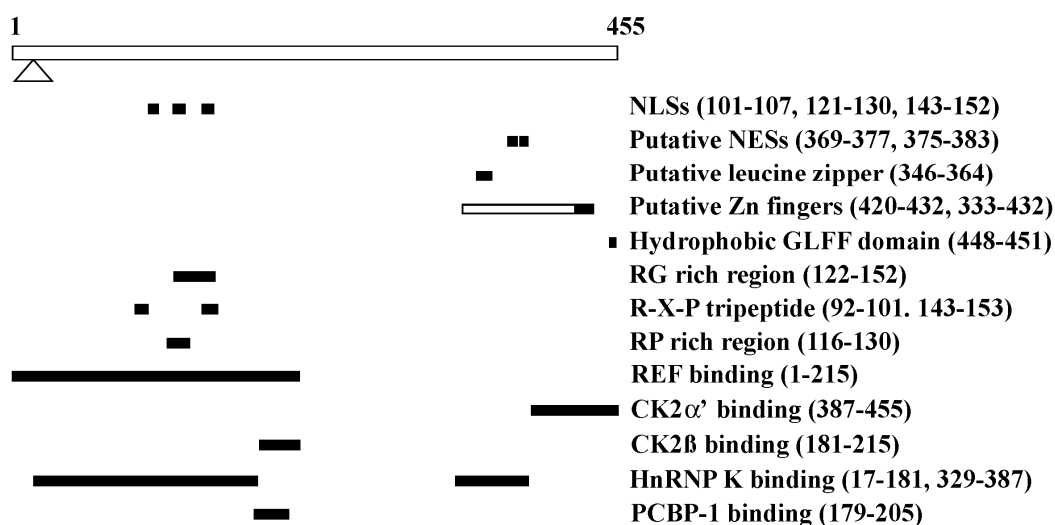
provide further insights into the nature of cellular mRNA processing and cellular gene regulation as they relate to oncogenesis leading to novel therapeutic interventions in infections and viral-induced cancers.

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## Figure Legends

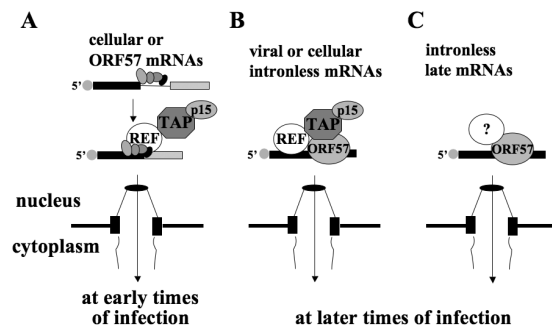
**Fig. 1**



### Schematic representation of the characterized and putative domains in the KSHV ORF57 protein

Amino acid residues are numbered on the top and domains (characterized and proposed) are shown below the schematic by solid black boxes and details of the residues provided on the side. NLS, nuclear localization signal; NES, leucine-rich putative nuclear export signal in ORF57 with moderate homology to a classical-type NES occurs at the C-terminus [L<sub>(369)</sub>NFRGGLLL<sub>(377)</sub> and L<sub>(375)</sub>LLAFVVLTI<sub>(383)</sub>]; RGG type box, arginine and glycine-rich region; R-X-P rich, arginine and proline rich region required for RNA binding; Zn finger, domain shown to chelate Zinc, two putative zinc finger-like motifs occur in the C-terminal domain of ORF57 based on amino acid similarity (C<sub>(333)</sub>-X<sub>89</sub>-H<sub>(423)</sub>-X<sub>3</sub>-C<sub>(427)</sub>-X<sub>4</sub>-C<sub>(432)</sub> or, H<sub>(423)</sub>-X<sub>3</sub>-C<sub>(427)</sub>-X<sub>4</sub>-C<sub>(432)</sub>), C terminus required for ORF57 self-interaction; GLFF motif (glycine-leucine-phenylalanine-phenylalanine), could be involved in ORF57-mediated *trans*-activation of viral genes.

**Fig. 2**



### Alternative export model in Herpesvirus infected cells

(A) At very early time points in infection intron containing immediate early viral mRNAs (such as ORF57 and major transactivator ORF50) could use the pathway utilised by cellular mRNAs for their export to get outside the nucleus for protein expression. (B) Once immediate early protein KSHV ORF57 is expressed, it recruits a minimal exon-junction complex (consisting of REF/Aly protein) and the TAP/p15 heterodimeric nuclear export receptor, (used principally by cellular mRNAs) to provide viral mRNAs access to cellular export pathway. (C) At the later stages of infection when viral genes are expressed in abundance and host cell mRNAs are inhibited and cellular protein synthesis is shut down, ORF57 or its homologues could themselves act as export receptors for either general viral mRNA export or export of a specific late class of mRNAs. Pathways shown in A and B are TAP-mediated and can be blocked by constitutive transport elements (CTE) from simple retroviruses shown to compete with TAP-mediated export but, theoretically, the pathway shown in C would have no effect on viral protein –mediated mRNA export by addition of CTE or the export receptor CRM1 inhibitor, leptomycin B.

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